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Multiparametric photoacoustic microscopy of the mouse brain with 300-kHz A-line rate

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Abstract. Enabling simultaneous high-resolution imaging of the total concentration of hemoglobin (C_{Hb}), oxygen saturation of hemoglobin (sO_2), and cerebral blood flow (CBF), multiparametric photoacoustic microscopy (PAM) holds the potential to quantify the cerebral metabolic rate of oxygen at the microscopic level. However, its imaging speed has been severely limited by the pulse repetition rate of the dual-wavelength photoacoustic excitation and the scanning mechanism. To address these limitations, we have developed a new generation of multiparametric PAM. Capitalizing on a self-developed high-repetition dual-wavelength pulsed laser and an optical–mechanical hybrid-scan configuration, this innovative technique has achieved an unprecedented A-line rate of 300 kHz, leading to a 20-fold increase in the imaging speed over our previously reported multiparametric PAM that is based on pure mechanical scanning. The performance of the high-speed multiparametric PAM has been examined both *in vitro* and *in vivo*. Simultaneous PAM of microvascular C_{Hb} , sO_2 , and CBF in absolute values over a ~ 3 -mm-diameter brain region of interest can be accomplished within 10 min. © 2016 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.NPH.3.4.045006]

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1 Introduction

Capable of providing label-free, comprehensive, and quantitative characterization of cerebral hemodynamics at the microscopic level, photoacoustic microscopy (PAM) has attracted increasing attention in neuroimaging.¹ A recent research focus is to combine the multiple PAM-measured hemodynamic parameters—including the total concentration of hemoglobin (C_{Hb}), oxygen saturation of hemoglobin (sO_2), and cerebral blood flow (CBF)—for quantifying the cerebral metabolic rate of oxygen (CMRO₂) at the microscopic level.^{2,3} To this end, we have developed multiparametric PAM, which enables simultaneous imaging of C_{Hb} , sO_2 , and CBF at the same spatial scale.⁴ However, the speed of multiparametric PAM has been severely limited by the pulse repetition rate of the dual-wavelength photoacoustic excitation and the scanning mechanism. By employing two commercially available high-repetition single-wavelength lasers with distinct pulse durations (3 ns and 3 ps) and a customized water-immersible microelectromechanical system (MEMS) scanning mirror, Yao et al.⁵ have markedly improved the speed of PAM and demonstrated functional imaging of the mouse brain with 100-kHz A-line rate. Although encouraging, this technique is not applicable for microscopic imaging of CMRO₂ because CBF—unlike the other two parameters—can only be measured at selected locations. Moreover, the pulse-duration-based measurement of sO_2 requires high-laser pulse energy (>400 nJ) to induce transient saturation in the optical absorption of blood hemoglobin, exceeding the safety limit recommended by the American National Standards Institute.⁶

Here, we report on an implementation of multiparametric PAM with an unprecedented A-line rate of 300 kHz. This

technique sticks to the established spectroscopic measurement of sO_2 , which requires much lower pulse energy (<100 nJ) than the saturation-based approach. To allow for high-repetition dual-wavelength excitation, we have developed a 300-kHz laser capable of pulse-by-pulse switching between 532 and 558 nm. To boost the imaging speed while retaining the unique capability of simultaneously mapping C_{Hb} , sO_2 , and CBF at the microscopic level, we have implemented optical–mechanical hybrid scan to acquire multiple cross-sectional scans (i.e., B-scans) in parallel. The performance of this new generation of multiparametric PAM has been examined in phantoms and the mouse brain.

2 Materials and Methods

2.1 Experimental Setup

As shown in Fig. 1, the high-speed multiparametric PAM utilizes an ytterbium-doped fiber laser (GLPM-10-Y13, IPG Photonics; wavelength: 532 nm; pulse repetition rate: tunable between 10 and 300 kHz; and pulse duration: 1.25 ns). The laser beam passes through an electro-optical modulator (EOM; 350-80, Conoptics) and a half-wave plate (HWP; WPH05M-532, Thorlabs) before being expanded by a lens pair (LA1213-A and LA1608-A, Thorlabs). When a high voltage (260 V) is applied to the EOM, the polarization of the incident beam is rotated to the vertical direction by the EOM and HWP. Thus, the expanded beam is reflected by a polarizing beam splitter (PBS; PBS121, Thorlabs) and then coupled through a microscope objective (M-10X, Newport) into a 5-m-long polarization-maintaining single-mode fiber (PM-SMF; F-SPA, Newport) for stimulated Raman

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Fig. 2 Scanning mechanism and measurement principle. (a) Scanning scheme of the optical–mechanical hybrid scan. (b)–(d) Correlation, statistical, and spectral analyses of CBF, C_{Hb} , and sO_2 , respectively. HbO_2 , oxyhemoglobin; HbR , deoxyhemoglobin.

(532 and 558 nm), sO_2 can be simultaneously quantified. As shown in Fig. 2(d), the absorption coefficients of oxy- and deoxyhemoglobin are nearly identical at 532 nm but distinct at 558 nm. Thus, sO_2 can be estimated using spectral decomposition.¹⁶

2.3 Animal Preparation

A male CD1 mouse (17 weeks old) was used for the *in vivo* study. For optimal imaging quality, the mouse skull above the right somatosensory cortex was thinned to $\sim 100\ \mu\text{m}$ prior to the PAM experiment, forming a circular imaging window with a diameter of $\sim 3\ \text{mm}$. Throughout the PAM experiment, the mouse was maintained under anesthesia with 1.0% to 1.5% isoflurane and the body temperature was kept at 37°C using a temperature-controlled heating pad (EW-89802-52, Cole-Parmer; SRFG-303/10, Omega). All experimental procedures were carried out in conformity with the laboratory animal protocol approved by the Animal Care and Use Committee at the University of Virginia.

3 Results and Discussion

3.1 Stability of the 558-nm Output

In stimulated Raman scattering, the Stokes light has a nonlinear dependence on the pump. Thus, small fluctuations in the 532-nm pump can be amplified when it is Raman shifted. To check the stability of the 558-nm output, we continuously monitored the pulse energy for 660 s and the average power for 120 min using the high-speed photodiode and a power meter (S120C, Thorlabs), respectively. Prior to the monitoring, the coupling efficiency of the 532-nm incident beam to the PM-SMF was maximized and the incident pulse energy was set to $1\ \mu\text{J}$, which led to a maximal 558-nm output of 220 nJ. The fluctuation of the room temperature was controlled within 1°C to avoid possible disturbance to the 532-nm laser and the PM-SMF. As shown in Fig. 3, the relative standard deviations of the Raman pulse energy and power were measured to be 0.76% and 1.42%, respectively. Such small fluctuation could be easily compensated for by the high-speed photodiode monitoring during *in vivo* experiments.

Fig. 3 Stability of the 558-nm Stokes output. (a) Short-term monitoring using high-speed photodiode. (b) Long-term monitoring using power meter.

3.2 Optical Resolution and Acoustic Focal Zone

The optically defined lateral resolution of the multiparametric PAM was examined by imaging a resolution target (R1DS1P, Thorlabs). As shown in Fig. 4(a), the sixth element of Group 7 was clearly resolved. By fitting the experimentally measured modulation transfer function (MTF) to the theoretical MTF of a perfect optical system,¹⁷ we estimated the cutoff spatial frequency to be 259.7 line pair/mm, which corresponded to a lateral resolution of 3.9 μm .

For the optical-mechanical hybrid scan, the optical scanning must be confined within the focal zone of the ultrasonic transducer to ensure sufficient signal-to-noise ratio. The acoustic focus in the transverse plane was experimentally characterized by 2-D optical scanning of a homogeneous black tape over an area of 65 μm by 65 μm . As shown in Fig. 4(b), the circular region with high-photoacoustic amplitudes corresponded to the acoustic focal zone. Gaussian fitting of the cross-sectional profiles [dashed lines in Fig. 4(b)] revealed the acoustic focal diameters along the X- and Y-axis to be 45 and 39 μm , respectively [Fig. 4(c)]. Thus, the voltage applied to the galvanometer scanner was carefully adjusted to spread the 20 pixels over the 40- μm range of optical scanning. It is worth noting that the non-uniform detection sensitivity within the 40- μm acoustic focal zone, as shown in Figs. 4(b) and 4(c), was mathematically compensated for before the quantification of C_{Hb} , sO_2 , and CBF.

3.3 Measurable Flow Range

The measurable flow range of the multiparametric PAM is codeetermined by the pulse repetition rate of the laser and the B-scan rate.¹⁴ Before, *in vivo* applications, this range was examined in a

Fig. 5 Examination of the measurable flow range in blood phantom.

vessel-mimicking phantom.^{14,18} Specifically, deliberated bovine blood (910, Quad Five) in a plastic tube (56514, United States Plastic Corporation) was driven to flow at 22 different preset speeds by a syringe pump (NE-300, Pump System). The flow speeds of the bovine blood were measured by the high-speed multiparametric PAM and compared with the preset values. Linear regression analysis revealed a strong linear relationship ($R^2 = 0.98$) between the measured and preset flow speeds within the range of 0.2 to 8 mm/s (Fig. 5).

Similar to our previous multiparametric PAM,¹⁴ the upper limit of the measurable flow range is determined by the time interval between two contiguous A-lines in the same B-scan. The smaller the interval, the faster the decorrelation it can sample for the correlation analysis. In our current high-speed multiparametric PAM system, the average interval is ~ 0.24 ms due to the 2.1-kHz round-trip rate of the galvanometer scanner. This

Fig. 4 Optical resolution and acoustic focal zone. (a) Optically defined lateral resolution quantified using a resolution target. (b) Acoustic focal zone within the horizontal plane delineated by 2-D optical scanning of a black tape. (c) Acoustic focal diameters along the X- and Y-axis quantified by the full-width at half-maximum (FWHM) values of the Gaussian-fitted cross-sectional profiles.

Fig. 6 Simultaneous high-speed PAM of (a) C_{Hb} , (b) sO_2 , and (c) CBF (both speed and direction) in the mouse brain.

upper limit can be readily improved using a resonant galvanometer scanner, whose round-trip rate is as high as 12 kHz (e.g., 6SC12KA040-04Y, Cambridge Technology). With that, we can push the limit to ~ 50 mm/s. The lower limit is determined by the 5.7-ms correlation window, as shown in Fig. 2(b). The time window is not wide enough to fully record slow decays induced by extremely low speeds. This lower limit can be extended by reducing the B-scan rate at the expense of the imaging time.

3.4 High-Speed Multiparametric Photoacoustic Microscopy of the Mouse Brain *In Vivo*

Upon examining the performance of the high-speed multiparametric PAM *in vitro*, we further tested its *in vivo* performance in the mouse brain. As shown in Fig. 6, the C_{Hb} , sO_2 , and CBF of individual microvessels were simultaneously imaged over a ~ 3 -mm-diameter cortical region through the thinned-skull window. The pulse energies applied to the mouse brain were 85 and 95 nJ at 532 and 558 nm, respectively. The total image acquisition time was ~ 10 min.

The imaging speed of the hybrid scan-based multiparametric PAM is currently limited by the acoustic focal zone and is insufficient to visualize rapid CBF changes in response to the brain stimulation. Within the $40\text{-}\mu\text{m}$ focal diameter, 20 dual-wavelength B-scans are acquired with an average interval of $2\text{ }\mu\text{m}$, which is much smaller than the $10\text{-}\mu\text{m}$ B-scan interval used in our previous multiparametric PAM and the MEMS-based high-speed PAM.^{4,5} Expanding the focal diameter along the Y-axis to $200\text{ }\mu\text{m}$ by a cylindrically focused transducer will allow us to spread the 20 B-scans with a $10\text{-}\mu\text{m}$ interval, thereby improving the speed by fivefold. Also, the correlation-based CBF measurement limits the B-scan rate to 0.88 mm/s . In applications where only vascular anatomy and sO_2 are of interest, the B-scan rate can be increased to 10 mm/s , which can further improve the imaging speed by an order of magnitude.

The axial resolution of the reported system is currently limited by the bandwidth of the ultrasonic transducer to $\sim 50\text{ }\mu\text{m}$. It cannot resolve overlapping vessels within this focal zone, which may affect the accuracy of the hemodynamic readouts. This limitation can be addressed by increasing the bandwidth of the ultrasonic detection.^{19,20}

4 Conclusion

We have developed a new generation of multiparametric PAM with an unprecedented A-line rate of 300 kHz. By integrating the high-repetition dual-wavelength Raman laser and the optical-mechanical hybrid scan, this technology innovation enables simultaneous high-resolution PAM of C_{Hb} , sO_2 , and CBF at a speed 20 times faster than that of the previous generation. Expanding the focal zone of the acoustic detection is expected to improve the imaging speed by another fivefold. Envisioned applications of this technique include longitudinal monitoring of hemodynamic and metabolic dysfunctions in ischemic stroke, traumatic brain injury, and Alzheimer's disease.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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